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DNA damage marker γ H2AX is a potential predictive marker for progression of epithelial dysplasia of the oral cavity

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Short running title: γ H2AX predicts progression of oral epithelial dysplasia

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Declaration of interests

We declare no competing interests.

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Abstract

Aims:

To evaluate the relationships between immunohistochemical markers related to cellular senescence, cell proliferation and histological grade of epithelial dysplasia of the oral cavity (OD). In addition, the predictive value of these markers for progression of OD was assessed.

Methods:

Retrospective immunohistochemical analyses were performed on 86 formalin fixed and paraffin embedded specimens of OD and oral squamous cell carcinoma (OSCC) for Ki67, γ H2AX, p53, p16, H3K9me3 and CycD1. Three separate areas representing the highest severity of OD on each slide were digitally annotated by two independent pathologists. Mean automated histoscores of the selected markers were generated and compared to that of age-matched healthy controls (n=24). Follow-up data of OD was retrieved and anonymised by a clinical team member and linked using unique participant identifiers. The median follow-up was 10.9 years (interquartile range: 10.1-11.5).

Results:

Ki67 ($p<0.0001$), γ H2AX ($p=0.03$) and p53 ($p=0.04$) were significantly increased with higher histological grade of OD. γ H2AX ($p=0.03$), but not histological grade of OD ($p=0.73$), was prospectively associated with disease progression. Using the median histoscore for γ H2AX (median histoscore = 17) as a cut-off, $\text{histoscore} \geq 17$ was associated with an increased risk of disease progression (HR=3.15, 95%CI 1.41-7.39, $p=0.0064$).

Conclusions:

Although proliferation marker Ki67, DNA damage/checkpoint markers γ H2AX and p53 were increased in higher grade of OD, only γ H2AX was predictive of disease progression. These observations may reflect the role of DNA replicative stress in the transformation from OD to OSCC. Larger studies should evaluate whether γ H2AX can be used as a predictive marker of OD.

Keywords: oral cavity, neoplasm, carcinoma in situ, cellular senescence, biomarkers, immunohistochemistry

Introduction

Although oral cancer accounts for only 3% of all cancer cases, its worldwide incidence has increased in recent years.¹ This increase has been associated with human papillomavirus infection, particularly the high-risk HPV-16 subtype, in cancers of the oropharynx.² Oral epithelial dysplasia (OD) is a known premalignant condition associated with oral squamous cell carcinoma (OSCC). Clinically, OD lesions appear as white or red patches (leukoplakia and erythroplakia respectively) occurring anywhere on the mucosal surfaces of the mouth.

Although histological assessment is the gold standard for diagnosis and the grade of OD is a key risk factor of malignant transformation, its assessment is subjective with substantial inter-observer variability.^{3, 4} Moreover, the majority of OD lesions do not progress to cancer: reported progression rates ranged between 6% and 36%.^{5, 6}

Although significant efforts have been made to improve the predictive value of histological grading, e.g. by combining DNA ploidy analysis with OD grading,⁷ accurate positive predictive markers of progression do not exist.^{8, 9} The mainstay treatment for OD remains regular surveillance and surgical resection,¹⁰ and patients with recurrence (10-20% of those with OD) may require multiple operations in an anatomically confined and complex region with significant impact on quality of life. Hence, improved understanding of the molecular mechanisms underlying the progression from OD to OSCC could help stratify risks of patients with OD and streamline their follow-up, with the potential to identify malignant transformation early, while minimising morbidity from active surveillance and repeat resections.¹¹

Key proteins in cell cycle and proliferation control have been evaluated as candidate biomarkers to predict progression in OD, with limited success.¹² Senescence is an irreversible form of growth arrest and, once it is established, cells are unable to express genes required for proliferation even when exposed to mitogenic growth factors. Hence,

senescence is a potent mechanism of tumour suppression, associated with human and mouse benign neoplastic lesions.^{11, 13} Senescent cells continue to remain metabolically active with characteristic large flattened morphology (at least in vitro), and also display characteristic changes in gene expression.¹⁴ At the molecular level, replicative senescence of human cells is triggered by telomere attrition, which is registered by the cell as DNA damage and results in the activation of the tumour suppressor gene *TP53*. During replicative senescence, the CDK inhibitor p16INK4a accumulates in the cell, inhibiting the CDK-mediated phosphorylation of pRB and contributing to cell cycle arrest. These two pathways are essential for senescence activation in human cells, and alterations of these pathways have been reported in large cohorts of OSCC and OD.¹⁵ In addition, senescence can be triggered by the presence of an activated oncogene in an otherwise normal cell.¹³

This study aimed to evaluate whether the expression of markers related to cellular senescence was altered in OD, compared to normal oral epithelium. In addition, the predictive potential of these markers was explored.

Materials and methods

Ethical approval

This study was approved by the local ethics committee of the West of Scotland on 3 December 2010 (REC reference number: 10/S0704/56).

Patient selection and follow-up data

Pathological archival specimen

Anonymised surplus formalin fixed and paraffin embedded (FFPE) specimens of OD and OSCC were retrospectively identified and retrieved from the NHS Greater Glasgow and Clyde Biorepository by a pathologist (DM). Follow-up data of OD was retrieved and anonymised by a member of the clinical team (NS) using unique participant identifiers.

Age-matched normal control samples

Age-matched healthy volunteers were identified at the head and neck surgical outpatient clinic of the Queen Elizabeth University Hospital (Glasgow, the United Kingdom) by a consultant surgeon (JDM) who provided routine care of the volunteers. Study participation was voluntary and written informed consent was obtained from each volunteer.

Biopsy procedure for healthy age-matched volunteers

A single 4mm disc of normal oral mucosa was obtained from each volunteer using a punch biopsy device after infiltration of a local anaesthetic. Standard antiseptic technique was followed. All biopsy specimens were fixed in neutral buffered formalin and embedded in paraffin before immunohistochemical staining.

Immunohistochemistry

Histology slides were stained for markers of proliferation (Ki67, CycD1), DNA damage/checkpoint markers (γ H2AX), cellular senescence (p53 and p16), chromatin structure (trimethyl-Histone H3 (Lys9) [H3K9me3]) and negative control (IgG) according to established protocols of the Histological Services at the Cancer Research UK Beatson Institute, with appropriate positive and negative controls. All staining was done on a Dako Autostainer Link48 autostainer (Dako, Cambridge UK).

The following staining conditions and antibodies were used in this study: Ki67 (RM-9106, Thermo Scientific, Clone SP6) at 1 in 200; γ H2AX (9718, Cell Signaling, Clone 20E3) at 1 in 50; p53 (M7001, Novacastral, Clone DO7) at 1 in 1000; p16 (725-4713, Roche, Clone E6H4) was used as received, H3K9me3 (07-442, Millipore, polyclonal) at 1 in 300; CycD1 (M3635, Dako, Clone SP4) at 1 in 50; M7023 (negative control, Sigma-Aldrich, polyclonal) at 1 in 9000. Apart from p16 and CycD1 staining, all staining was performed after heat-induced epitope retrieval in citrate buffer at pH6. For p16 and CycD1 staining, antigen retrieval was performed in Tris-EDTA at pH9 and EDTA pH 8, respectively.

Interpretation of immunohistochemical assays

Stained slides were digitised (Hamamatsu NanoZoomer NDP, Hamamatsu Photonics, Welwyn Garden City, UK), reviewed and analysed using Slidepath Digital Image Hub V4.0.7 (Leica Microsystems, Milton Keynes, UK).

Histological grade of OD and qualitative assessments of the histology slides were performed by two collaborating pathologists (KH and CEM), who were blinded to the histological grade of the original diagnostic report at the time of their assessments. As discrete areas of OD often develop within a background of normal tissue, three separate areas representing the highest severity of OD on each slide were digitally annotated based

on hematoxylin and eosin stained specimen slides before the assessment of other immunohistochemical markers. For specimens from normal healthy controls, three random areas of normal epithelium were annotated.

Automated Histoscores [$0 \times \% \text{ negative cells} + 1 \times \% \text{ weakly stained cells} + 2 \times \% \text{ moderately stained cells} + 3 \times \% \text{ strongly stained cells}$] of the annotated areas were then generated by digital image analysis. Mean values of the histoscores generated from the three separate areas of each slides were used for statistical analysis of the immunohistochemical markers assessed. Each histoscore has a range of possible scores between 0 and 300. The algorithms used were unable to generate histoscores for <5% of samples.

Human papillomavirus (HPV) status

The HPV status of p16-positive specimens was evaluated by HPV 16/18 polymerase chain reaction (PCR) at the Queen Elizabeth University Hospital (Glasgow, the United Kingdom) according to established protocol.

Statistical analysis

Statistical tests were performed using Prism 6 (GraphPad software, San Diego, CA, US). Differences between groups were analysed using Kruskal-Wallis and Mann-Whitney U tests, as the data were not normally distributed. Significance was considered with a p-value below 0.05. The level of statistical significance is indicated using asterisks (* $p < 0.05$ and **** $p < 0.0001$). When appropriate, data were presented as median and interquartile range (IQR). Survival analysis was performed using Log-rank test; hazard ratio (HR) and 95% confidence interval (95%CI) were presented in addition to p-value.

Results

The relationships between grade of disease and biomarkers assessed

For the archival specimens (n=86), only age and histological grade at diagnosis were available. The median age of diagnosis was 64.6 years old (interquartile range: 53.9-71.5). Twenty-four (27.9%) of these archival specimens had mild OD, 17 (19.8%) had moderate OD, 32 (37.2%) had severe OD and 13 (15.1%) had OSCC. Automated histoscores of the assessed immunohistochemical markers were compared by grade of disease, compared to 24 normal age-matched controls (Table 1, Figure 1 and supplementary Figure 1). With respect to p16 expression, seven of the 32 severe OD specimens (21.9%) expressed a high level of p16. Given the known link between HPV status and p16, their HPV status was evaluated. Five were HPV positive (71.4%, all HPV-16 positive), one was negative (14.3%) and one had too little remaining DNA for assessment (14.3%).

More significantly, the expression of Ki67 ($p<0.0001$), γ H2AX ($p=0.03$) and p53 ($p=0.04$) was significantly increased with higher histological grade of OD. The expression of these biomarkers plateaued or reversed in OSCC.

The relationships between biomarkers assessed and disease progression

The median follow-up was 10.9 years (IQR: 10.1-11.5). Of the 73 patients with OD, 18 improved and were discharged from follow-up (24.7%), 4 progressed to higher grade of dysplasia (5.6%) and 19 progressed to develop malignancies (26.0%). For the 23 patients with disease progression, the median time to progression was 6.0 years (IQR: 4-6.9). There was no difference in the rate of progression by grade of dysplasia (supplementary figure 2, $p=0.73$).

The relationships between assessed biomarkers and disease progression in patients with OD were evaluated (Table 2). Two of the 7 patients with a high level of p16

(28.6%) progressed to develop OSCC, of whom only one had confirmed HPV infection. Most interestingly, increased γ H2AX was associated with disease progression (Table 2, $p=0.03$). Using the median histoscore for γ H2AX (median histoscore = 17) as a cut-off, histoscore \geq 17 was associated with an increased risk of disease progression (Figure 2; HR=3.15, 95%CI 1.41-7.39, $p=0.0064$).

Discussion

Main findings, strengths and limitations

Higher histological grades of OD were significantly associated with increased Ki67, γ H2AX and p53, and subsequently plateaued or reversed in patients with malignancy, similar to changes observed in patients with Barrett's oesophagus.¹⁶ Moreover, γ H2AX, which predictive value has not been thoroughly evaluated⁶, was associated with disease progression.

Although p16 expression is an established marker of senescence, infection with HPV upregulates expression of non-functional p16 due to the loss of pRB function, which is unlikely to be associated with senescence. Indeed, in patients with severe OD, the majority of specimens with high p16 expression had confirmed HPV infection. Consistent with previous reports that only a small proportion of patients with OSCC (<5%) were infected with HPV¹⁷ and that p16 was a poor predictive marker of OD^{8, 18}, p16 was not associated with increasing histological grade of OD nor progression in this cohort. Other novel senescence markers assessed in this study did not show significant changes in expression by grade of OD or progression status.

This study is one of the few studies reported to date that included a normal age-matched control group.^{8, 19} This highlights the feasibility of establishing a cohort of normal healthy control, which is important for the development of clinically relevant biomarkers.²⁰ In addition, this study also procured sufficiently long follow-up, and the rate of progression

to malignancy was similar to that in previously reported studies (26%).^{5, 6} However, the number of participants included in this exploratory study was small. It had limited power to detect small changes in the expression of the biomarkers assessed. Moreover, multiple comparisons could increase the probability of type I errors. Therefore, larger studies would be required to validate the associations observed.

Recommendations for future studies

The expression of proliferation markers Ki67, DNA damage/checkpoint markers γ H2AX and p53 was significantly higher with increasing grades of OD, which may aid the determination of histological grade of OD. The significance of the observed patterns of expression of Ki67, γ H2AX in and p53 in OSCC is unclear. Including normal controls in future biomarker studies will help confirm the patterns observed.

While grade of dysplasia did not predict progression in this cohort of patients, γ H2AX overexpression was significantly associated with disease progression, with p53 showing a similar trend. It was previously proposed that DNA replication stress, consistent with γ H2AX overexpression observed, may promote genomic instability that may encourage progression from OD to OSCC.²¹ Future studies could further evaluate whether the combination of these two common immunohistochemical markers, γ H2AX and p53, may better predict the progression of OD.

Conclusion

Although proliferation markers Ki67, DNA damage/checkpoint markers γ H2AX and p53 were increased in higher grade of OD, only γ H2AX was associated with disease progression, which may indicate the potential of DNA replicative stress in the transformation from OD to OSCC. Larger studies could evaluate whether γ H2AX could be used as a predictive marker of OD.

Contribution to authorship

PDA, KH, JDM and EYL contributed to the conception of the research question. EYL directed the experiments and, together with CN, contributed to the experiments performed. JDM collated samples from volunteers and oversaw the clinical database. DM identified and released the relevant pathological archive specimens. NS contributed to retrieve clinical data. CO and CB contributed to generate the automated histoscores. KH and CEM provided histological assessments of this project. EYL wrote the manuscript. All authors revised the manuscript and approved the final version.

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1 Table 1: Automated histoscores by grade of disease, compared to normal healthy control (n=110).

2

	Normal	Mild OC	Moderate OC	Severe OC	OSCC	p-value
Ki67	58.3 (47.2-69.5)	56.5 (45.1-74.1)	78.0 (47.0-105.2)	94.3 (82.3-121.0)	71.3 (40.0-110.0)	<0.0001
γH2AX	11.3 (7.7-19.2)	13.8 (8.5-17.9)	16.0 (7.7-41.4)	25.5 (11.3-62.3)	27.0 (4.0-46.2)	0.03
p53	13.5 (4.4-22.2)	37.9 (12.7-43.7)	39.7 (14.4-95.3)	17.7 (3.0-111.3)	19.0 (2.3-75.5)	0.04
p16	0 (0-1.0)	0.3 (0-2.8)	0 (0-1.0)	0 (0-5.0)	0 (0-0.7)	0.59
H3K9me3	74.0 (58.3-95.1)	76.5 (56.6-111.3)	77.7 (17.5-105.9)	66.2 (36.1-108.2)	63.3 (39.3-94.0)	0.68
CycD1	31.5 (16.9-67.5)	23.5 (8.3-51.3)	29.0 (9.5- 71.8)	42.2 (8.9-76.8)	56.3 (30.4-82.9)	0.19
M7023 (Negative control)	0 (0-0.2)	0.3 (0-1.3)	0 (0-0.3)	0.7 (0-1.6)	0 (0-2.5)	0.10

3 Data presented as median (IQR)

4

5

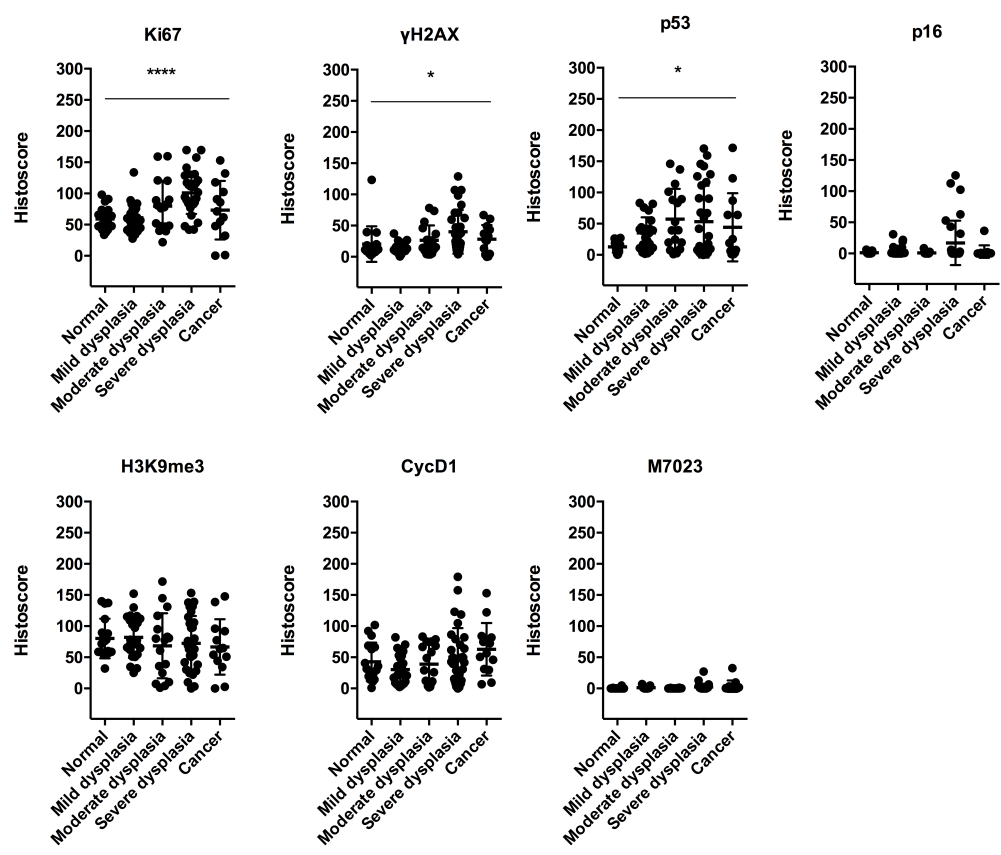
1 Table 2: Automated histoscores of patients with OD by progression status (n=73).

2

	No progression n=50	Progression n=23	p-value
Ki67	81.0 (47.8-114.7)	83.0 (60.3-101.7)	0.65
γH2AX	15.3 (8.6-26.9)	25.5 (14.5-56.0)	0.04
p53	28.9 (7.8-56.3)	69.2 (9.9-95.9)	0.15
p16	0.2 (0-3.0)	0 (0-0.7)	0.19
H3K9me3	70 (36.5-105.1)	80 (51.2-112.0)	0.55
CycD1	28.5 (7.9-58.7)	49.0 (12.7-75.3)	0.11
M7023 (Negative control)	0.3 (0-1.3)	0 (0-0.9)	0.54

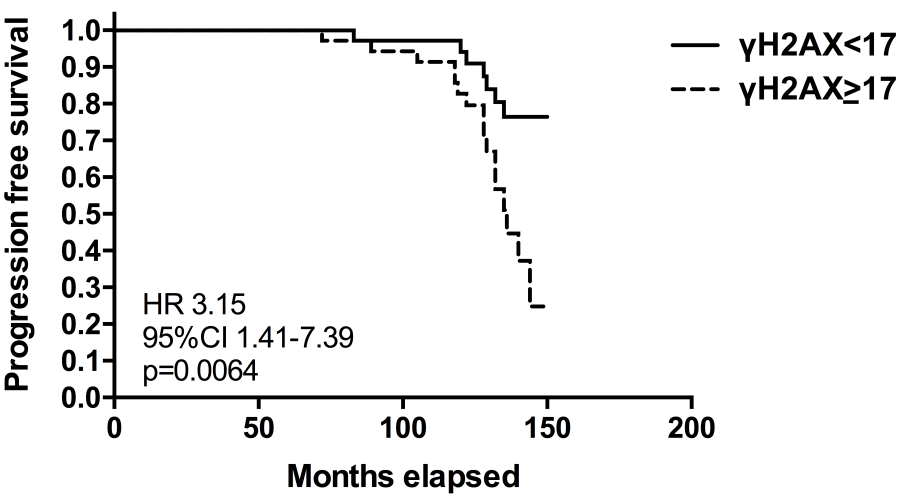
3 Data presented as median (IQR)

1 Figure 1: Summary of automated histoscores by grade of disease, compared to normal
2 healthy control (n=110).
3



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1 Figure 2: Kaplan-Meier curve of progression-free survival (PFS) in patients with OD.
2



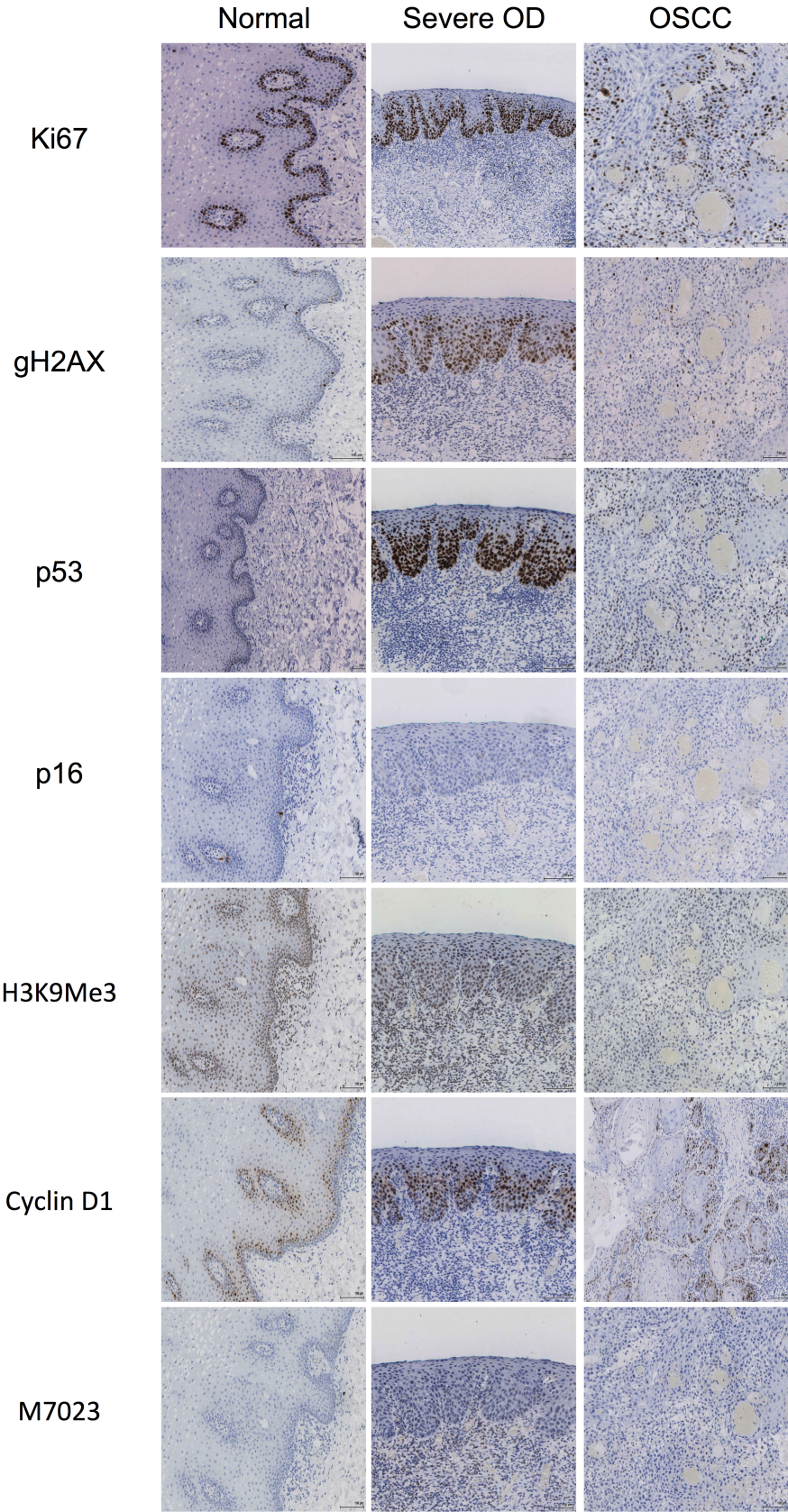
	Event/n*	Median PFS (months)
$\gamma\text{H2AX}<17$	7/36	Indefinite**
$\gamma\text{H2AX}\geq 17$	16/36	136

*Histoscore of γH2AX was unavaialbe for one specimen

**Fewer than half in this group had progressed at their last follow-up visits

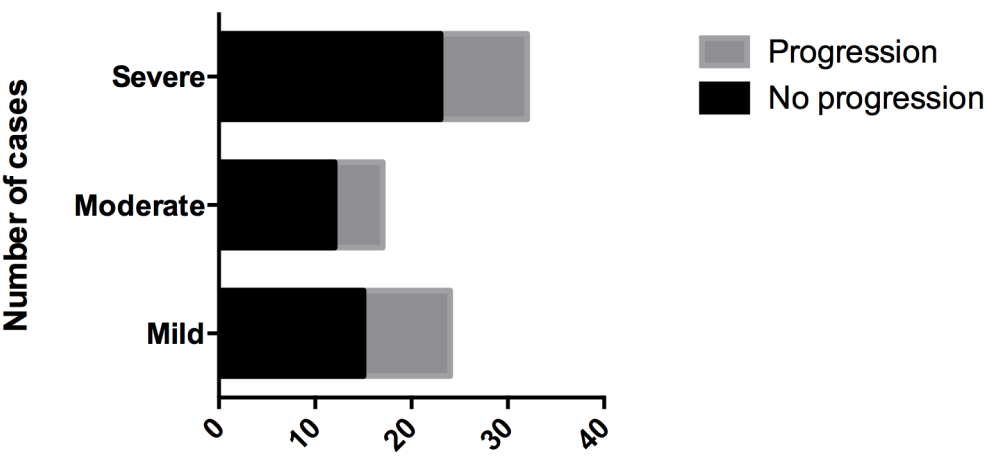
3
4

1 Supplementary figure 1: representative images of the immunohistochemical staining by
2 grade of disease, compared to normal healthy control (n=110).
3



4
5

1 Supplementary figure 2: Progression status by grade of OD.
2



	Mild (n=24)	Moderate (n=17)	Severe (n=32)
No progression	15 (62.5)	12 (70.6)	23 (71.9)
Progression	9 (37.5)	5 (29.4)	9 (28)

Data presented as number (%)

3
4